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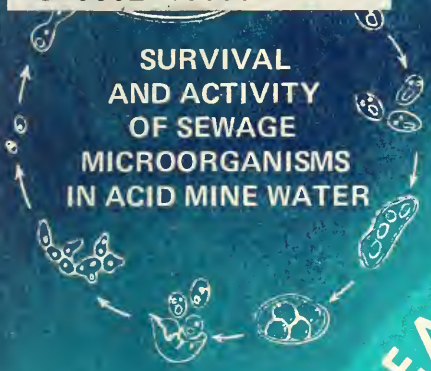
RESEARCH REPORT 1

SURVIVAL AND ACTIVITY OF SEWAGE MICROORGANISMS IN ACID MINE WATER



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Survival and Activity of Sewage Microorganisms in Acid Mine Water

by
Raymond C. Kralovic
and
H. A. Wilson

RESEARCH REPORT 1

This publication reports results of work supported in part by funds authorized under the Water Resources Research Act of 1964 (PL 88-379) which is administered by the Office of Water Resources Research, United States Department of the Interior.

WATER RESEARCH INSTITUTE
Appalachian Center
WEST VIRGINIA UNIVERSITY
Morgantown
1969

Foreword

The Board of Governors of West Virginia University established the Water Research Institute on September 21, 1963. On May 20, 1965, the Institute became the West Virginia Water Resources Research Center under the Water Resources Research Act of 1964 (PL 88-379). The Act provides for a continuing allotment of funds to the Institute and is administered by the Office of Water Research of the United States Department of the Interior.

The fundamental aim of the Water Research Institute is to obtain the knowledge needed to gain the greatest benefit from the water resources of West Virginia. To aid in meeting this aim, the Institute reports the results of research done under its program, and to meet different requirements, the research results are published in four report series—the Bulletin, Research Report, Journal Report, and Information Report. The Bulletin is used to report the final results of projects and programs. The Research Report is for early publication of special, highly technical findings of project research. The Journal Report is used to report research results in scientific journals and other outside publications. The Information Report is used for collections of short papers, compilations of data, and other such information that is not suitable for other reports.

This first Research Report covers work done as part of Project A-002-WVA, which was supported chiefly by allotment under the Water Resources Research Act under Allotment Agreements 14-01-0001-707, -819, -994, -1417 and 1869. Substantial support was also given by the Agricultural Experiment Station and the Department of Plant Pathology and Bacteriology, West Virginia University. Because of the support and cooperation of the Experiment Station, the report is being also designated Bulletin 572 of the Station. General administrative support was given by West Virginia University, the Appalachian Center, and the Office of Research and Development. Cooperation on Institute work extends widely, especially to institutes in other states, and to all who aid us, we gratefully acknowledge the cooperation.

During the time the research was being done, Raymond C. Kralovic was a graduate research assistant of the Water Research Institute, and many of the results of this report were previously reported in his Master of Science Degree thesis. Dr. Harold A. Wilson is the Principal Investiga-

tor of Project A-002-WVA and also Professor of Bacteriology, Department of Plant Pathology and Bacteriology, and Bacteriologist, West Virginia Agricultural Experiment Station.

Work continues on the project and additional results will be published.

CHESTER L. DODSON
Director
Water Research Institute
January 1969

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CONTENTS

Foreword iii

Abstract vii

Introduction 1

Literature review 1

Types of microorganisms in raw sewage 2

Effects of pH on microorganisms 3

Influences of low temperatures on growth of microorganisms ... 5

Biochemical oxygen demand of raw sewage 5

Materials and Methods 6

Results and discussion 8

Survival of microorganisms 9

Methods 9

Results 10

Discussion 13

Selectivity 14

Method 14

Results 14

Discussion 14

Oxygen demand 15

Influence of pH upon oxygen demand 16

Method 16

Results 16

Effects of acid mine water and sulfuric acid on raw-sewage microorganisms 16

Method 16

Results 17

CONTENTS (Continued)

Effect of partial neutralization of acid mine water upon oxygen demand 17

Methods 17

Results 19

Methods 19

Results 19

Oxygen demand using bacteria or yeasts 21

Methods 21

Results 21

Discussion 21

Microbial isolates and observations 24

Isolates 24

Methods 24

Results 24

Methods 24

Results 25

Observations 25

Discussion 26

Conclusions 27

Literature cited 29

FIGURES

1. Effects of acid mine water on the number of microorganisms in raw sewage mixed 1:1 with acid mine water. Incubation at 22 C . 10
2. Growth of raw sewage microorganism at 4 C and 22 C in raw sewage acid mine water mixtures adjusted to pH 3.5 11
3. Raw sewage acid mine water mixtures 1.2. Initial pH 2.65 13
4. The numbers and types of microorganisms recovered from raw sewage using a medium adjusted to various pH levels 15
5. Oxygen demand of raw sewage - acid mine water mixtures at various pH levels 17
6. Oxygen utilization by sewage microorganisms in raw sewage acid mine water mixtures at various pH levels 18
7. Oxygen utilization by sewage microorganisms in raw sewage - H_2SO_4 mixtures at various pH levels 18
8. Oxygen uptake of raw sewage - acid mine water and NaOH mixtures at various pH levels. 19
9. Comparison of the effects of neutralized and unneutralized acid mine water on the BOD of raw sewage 20
10. Comparison of BOD values obtained in sterile raw sewage - acid mine water mixtures inoculated with acid tolerant yeasts and bacteria 22

TABLES

1. Comparison of four different media in recovery of microorganisms from raw sewage - acid mine water mixtures at pH 2.9 9
2. Microbial survival in sewage - acid mine water mixtures (pH 3.5) . Incubated at 4 C and 22 C 12
3. Quantitative and qualitative estimates of microorganisms found in Monongahela River water 24
4. Quantitative and qualitative estimate of microorganisms in raw sewage - acid mine water and raw - sewage - H_2SO_4 mixtures at pH 3.5 and containing less than 0.5 per cent oxygen after 50 - hours incubation at 20 C. 26
5. Numbers of microorganisms in raw sewage - acid mine water mixtures at various pH levels after 120 - hours incubation at 20 C. 26

ABSTRACT

Numbers and types of bacteria decrease if the pH of sewage is lowered by addition of acid mine water. A rapid decrease occurs at 4 C and 22 C, but at 22 C microbial numbers increase after the initial decrease.

The biochemical oxygen demand (BOD) of sewage and acid-mine-water mixtures decreases as the pH of the mixture decreases. At pH 3.5, there is a lag period before much biochemical oxidation occurs.

Oxygen utilization is greater if yeasts are inoculated into a sterile sewage and acid-mine-water mixture than when bacteria are used. If together, utilization is greater than for either alone.

Qualitative and quantitative microbial estimations were made on Monongahela River water which contains domestic raw sewage and acid mine water. Yeasts were the dominant organisms if the plating medium was at pH 4.0, and bacteria were dominant if the medium was about pH 7.0.

The actually acid-tolerant bacteria that will grow in sewage and acid-mine-water mixtures at pH 4.0 probably represent only a few genera. Four such isolates were identified as *Bacillus* species. Although each had characteristics of both *B. coagulans* and *B. firmus,* characteristics were sufficiently different to prevent positive identification. Other isolates were identified as *Micrococcus candidus* and *Kurthia variabilis.*

Descriptors: Acid mine water, Sewage, Microbiology, Biochemical oxygen demand, Oxygen utilization, Acid-tolerant bacteria, Yeasts, Hydrogen ion concentration.

Survival and Activity of Sewage Microorganisms in Acid Mine Water[+]

Raymond C. Kralovic and H. A. Wilson

INTRODUCTION

Many streams in the coal mining regions of the United States receive drainage from mines. If coal seams are associated with iron polysulfides, the drainage from the mines may range from slightly to strongly acid, so that such drainage waters with a pH below 3.0 are not uncommon. Some of these streams also receive domestic sewage; effluents from septic tanks and primary stage sewage plants; industrial wastes, such as pickling liquor, from steel mills; and wastes from meat packing and dairy plants.

The decomposition of organic wastes in a stream that has a pH of approximately 7.0 is carried out by microorganisms. The final products are microbial protoplasm, carbon dioxide, water, and inorganic compounds such as those of nitrogen, phosphorus, and potassium.

On the other hand, as the pH level of a stream is lowered by acid mine drainage a change occurs in the microflora. Many bacteria cannot tolerate acid conditions below pH 4.0. In fact, many become inactive or are killed before a pH 4.0 is reached. Because of a smaller variety of microorganisms in sewage polluted-acid mine water streams, sewage decomposition in such streams will be different than in streams of approximately neutral pH.

This publication reports some of the data obtained from a study of the effects of acid mine water on sewage microorganisms.

LITERATURE REVIEW

No discussion of the formation of acid mine water or its economic or esthetic disadvantages will be made, since these topics are well covered in the literature (Anderson, 1964; Colmer and Hinkle, 1947; Hodge, 1938). This review will include only some of the references which directly pertain to the subject of this study,

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+Published with the approval of the Director of the Agricultural Experiment Station as Bulletin 572.

Roetman (1932) reported over 99 per cent reduction in the numbers of microorganisms in a mixture of raw sewage (95%) and acid mine water (5%) in a 24-hour period. Joseph and Shay (1952) however, discounted any belief that streams carrying sewage will be purified by the addition of acid mine water. They studied the effects of acid mine water on sewage organisms *in situ,* by sampling streams polluted with sewage (a) before they received acid mine water, (b) shortly after receiving acid mine water, and, (c) after the streams had carried the acid mine water for a considerable distance. All streams examined had a pH of 2.0 to 4.5.

Wilson, Hipke and Rogers (1965) studied the effect of pH on the growth of microorganisms in sewage. Citric acid-phosphate buffers were used to adjust sewage to various pH levels. Their results showed that as the pH of the sewage was decreased there was an increase in the lag period required before turbidity developed. A difference was observed in the microflora between pH levels above 5.5 and below 4.5. Rogers (1964) and Rogers and Wilson (1966) also reported that the numbers of viable organisms at pH levels below 5.6 became smaller as the pH decreased and at pH levels of 4.6 and 3.5 the numbers of viable bacteria were small compared to the numbers found at higher pH levels (7.8, 7.4, and 5.6). Carpenter and Herndon (1933) showed that a tremendous reduction in microbial numbers occurred in mixtures of sewage (90 or 95%) and acid mine water (5 or 10%) during the first three hours of incubation. Incubation was carried on for 119 days. After the initial reduction in the numbers of microorganisms there was an increase which reached a maximum at the end of 6 or 7 days and dropped until the end of the incubation period.

Types of Microorganisms in Raw Sewage

Joseph and Shay (1952) found *Escherichia coli* in all acid mine water sewage polluted streams studied. Rogers (1964) isolated 50 microorganisms from the Monongahela River, which receives both sewage and acid mine water. All isolates grew at pH 4.0; seven of them were identified as yeasts. The remaining 43 were bacteria of which 22 were gram negative rods. Two of these were identified as *Aerobacter aerogenes* and a third was placed in the genus *Escherichia.*

The presence of filamentous fungi in sewage polluted waters undoubtedly is of great importance when such waters also receive acid mine water. These organisms are known to be markedly acid tolerant or even acidophilic (Thimann 1963).

Studies *in vitro* by Rogers (1964) and Wilson, Hipke and Rogers (1965) showed the presence of yeasts in sewage adjusted to low pH levels. Wilson, Hipke and Rogers also showed that two yeasts (*Rhodotorula mucilaginosa* and *Trichosporon cutaneum*) were capable of metabolizing glucose at the low pH values obtained with acid mine water. They, too, concluded that yeasts may be of importance in the degradation of sewage in acid mine water environments.

Effects of pH on Microorganisms

Although a number of factors are responsible for the effects of acid mine water upon microorganisms, pH is undoubtedly the most important and influences the effects of other factors either directly or indirectly. Much has been written on the effects of pH on microorganisms, but little is known about the mechanisms through which these effects are achieved. Extremes of pH will kill bacteria directly through denaturing or hydrolyzing of cellular constituents (Hewitt, 1957).

In biological systems, extremes of pH levels seldom occur and are thus of minor importance. Intermediate levels of pH are much more important and must be considered in the simplest of media. Thimann (1963) states: "The small size and great mobility of the hydrogen ion undoubtedly render it of supreme importance in many chemical processes; in biological processes its importance is still greater because so many of the processes consist of a transfer of hydrogen from one molecule to another. The tendency of the hydrogen to dissociate from its combination thus determines the probability of the reaction." McCalla (1941) postulated that the toxic effect of the hydrogen ion is not caused by any direct lethal effect but by a displacing and holding of the absorption positions by nutritive ions. Lilly and Barnett (1951) expressed a similar view of the action of the hydrogen ion on fungal cells. Lichstein (1959) reported that in bacterial cells pH may influence the permeability of the cells to some nutrients essential for growth initiation. An example of such is the increased penetration of dicarboxylic acids into the bacterial cell at low pH levels.

Hydrogen ion concentration is known to effect the toxicity of other substances to cells, especially weak electrolytes. Dagley, Dawes, and Foster (1953) showed that for cultures of *Aerobacter aerogenes* and *Bacterium* (*Escherichia*) *coli* low pH was responsible for the increased toxicity of formic acid produced by the cells. It is well known that weak acids are often more toxic than strong ones at the same pH levels. This effect is attributed to the undissociated molecule of the weak acid which can

more easily penetrate into cells than ions (Stanier, Doudoroff and Adelberg, 1957).

It is not known whether the internal pH of bacterial cells is affected by the external pH (Lamanna and Mallette, 1957). In the larger cells of fungi the internal pH has been shown, using indicators, to be affected by external pH. These methods however, are not precise (Lilly and Barnett, 1951; Cochrane, 1958).

Changes in external pH may cause a change in the metabolism of cells. Neal *et al* (1965) reported that a change from pH 6.2 to 3.0 in the external medium caused, within a one-minute period, "a decrease of internal pH to 4.3" of suspended yeast cells. Lilly and Barnett (1951) cited the example of a fungus, *Sordaria fimicola,* which was made thiamine deficient by lowering the pH to 4.0. They suggested that the low external pH may have reduced the internal pH to where synthesis of thiamine was inhibited. Hewitt (1957) stated that changes in the pH of the culture medium of bacteria result in a change of the enzymic composition of the cells so that essential activities are maintained at a constant level. These changes will not occur if the bacteria are merely suspended in a medium of a different pH and without growth of the bacteria. He also reported that the changes which a bacterial cell undergoes in response to a change of external pH may be of a compensatory, adaptive or permanent nature. If the changes are compensatory the cells will revert to their normal pattern as soon as the pH is returned to the original level. Adaptive changes are characterized by changes in the enzyme systems of the cells and some time is required before the cells revert to their former pattern. The failure of the cells to revert results in permanent changes.

It is obvious from the above discussion that pH is capable of exerting or influencing major changes on the growth of microorganisms. There are a number of possible mechanisms through which pH may act and the probable site for this action is at or near the protoplasmic membrane of the cells. The effect of pH may occur independently of the cell membrane by preventing or causing the dissociation of molecules and thus controlling their absorption by the cells. Since pH is able to control the absorption of materials by cells, it is probable that it also is able to effect their internal pH. Changes in the internal pH are manifest in the influences of external pH on the enzymatic activity of cells. A more complete review of the literature is given by Rogers (1964) who concluded that the internal pH of the cells was affected by the external pH and that "the cell membrance appears to play an important role in maintaining a favorable internal pH over a wide range of external pH whereas the mem-

brane of an organism extremely sensitive to changes in pH may not be capable of this function."

Influences of Low Temperatures on Growth of Microorganisms

Bacteriologists in the past, according to Porter (1946), have paid little attention to the effects of low temperature on bacteria because of the general belief that cold is an excellent means of preventing putrefaction and decay. There are however a large number of ubiquitous bacteria able to metabolize actively at temperatures as low as 0 C and at reduced rates to −5 C. (Ingraham, 1962). Low temperatures, above freezing, are generally thought to have a preservative effect on bacteria.

Ingraham (1962) also points out that certain bacteria are susceptible to killing by sudden exposure to low temperatures without freezing. No explanation of this phenomenon is known. Low temperatures are shown to have a preservative effect on the viability of *Bacterium* (*Escherichia*) *coli* and *Bacterium* (*Salmonella*) *typhosum* cells in phosphate buffer solutions at pH levels of 3.5, 6.1, 7.1 and 8.0 (Cohen, 1922).

The general conclusion is that low temperatures generally have a protective effect on cells and preserve their viability longer than if they were stored at higer temperatures. This is true even when the cells are stored under unfavorable conditions such as in the presence of disinfectants in the medium. If the temperature is lowered beyond the freezing point many more factors will affect the preservation of cells compared with preservation above the freezing point, i.e., rate of ice formation, crystal size, and thawing.

Biochemical Oxygen Demand of Raw Sewage

Roetman (1932) reported that the biochemical oxygen demand (BOD) of acid mine water and sewage is practically zero. It must be pointed out however, that ferrous iron and sulfur compounds may interfere with this determination (Standard Methods, 1960).

Ruchhoft, Ettinger, and Walker (1940) studied the BOD of acid water (dilute sulfuric acid) and sewage mixtures. They reported that in acidified sewage (pH 2.0 to 3.0) purification proceeded rapidly after a long lag phase. This was followed by a period in which further purification was slow. The amount of deoxygenation in acidified sewage was only about 65.5 per cent of that which occurred in sewage of the normal pH range (7.0 to 8.0). They suggested that acid may not be the only biological inhibitor present in acid mine streams.

Other methods for determining BOD may be more useful than the Winkler method, especially where interfering substances may be present.

Caldwell and Langelier (1948) adapted a Warburg apparatus for determining the BOD value of sewage. They reported that this apparatus has an advantage over the standard dilution method in that with it they were able to obtain values in 24 hours at 25 C equal to 75 per cent of standard 5-day values. The reproducibility is reported to be about the same as dilution methods. Umbreit, Burris, and Stauffer (1957) report that manometric techniques are useful where a complete demand is desired, since a reading can be obtained at any time without disturbing the system. A disadvantage of such techniques is that a large number of samples cannot be run easily because of the amount of apparatus and space required. Standard Methods (1960 but not 1967) gave tentative approval of a manometric technique using a Warburg apparatus for the study of the oxidation characteristics of wastes, such as oxidation rate constants, the effects of nutrient addition, pH adjustment, use of adapted seed, effect of waste concentration, and amenability of wastes to biological treatment.

MATERIALS AND METHODS

Before an interceptor sewer was installed, the sewage used in these studies was collected from a sewer outlet along the Monongahela River behind the West Virginia University Field House. After March 1966, the sewage was obtained from a combination sewer in Westover, West Virginia. Inasmuch as the weather was droughty, little or no storm water was being carried by the combination sewer.

All collections were made in glass bottles which were thoroughly washed and then rinsed with the sewage being collected before the sample was taken. Monongahela River water was obtained at the bottom of Walnut Street in Morgantown, West Virginia, and at the end of University Avenue, at the old ferry landing, Star City, West Virginia. The acid mine water was collected from a mine pump near Osage, West Virginia. A heavy flow from the pump was present at all times collections were made. Acid mine water and river water were collected in thoroughly cleansed bottles rinsed with the material being collected before the samples were taken. Each container of acid mine water was filled to overflowing and then tightly stoppered with a rubber stopper. The pH of all samples, media, and other materials was determined electrometrically.

Inasmuch as ferrous iron, and perhaps other elements and compounds, interfere with the titration procedures for biochemical oxygen

demand (BOD) two other methods were used in this study. Oxygen utilization was determined using a Beckman Oxygen Analyzer Model 777*. The sensor was easily adapted to fit into the mouth of a standard ground glass stoppered BOD bottle by fitting a short piece of the proper size rubber tubing onto the sensor. A magnetic stirring bar was placed in each bottle, and whenever a determination was taken, the bottle was placed upon a magnetic stirrer. In this manner the liquid within the bottle was rapidly moved past the sensor when the determination was being made. The data were recorded as per cent of oxygen saturation in the mixtures at regular intervals.

Oxygen demand determinations of the sewage mixtures, using a Hach manometric BOD apparatus**, were also made. The results are recorded as parts per million (ppm) of oxygen utilized.

The standard medium used in these studies for the enumeration of the microorganisms as well as for stock culture maintenance was Plate Count Agar (Difco) ***. A liquid medium of the same formulation (less agar) also was used.

Media of low pH (4.0) were prepared by either of two precedures. Known amounts of rehydrated Plate Count Agar were autoclaved and to these a definite volume of a sterile weak solution of sulfur acid was added. The quantity of sulfuric acid required had been previously determined. This technique yielded a medium ± 0.2 pH unit of the desired level.

Another procedure was to prepare a double strength medium from the various ingredients in Plate Count Agar, except for the agar, and adjust the pH of the liquid to a level 0.2 pH unit below the desired level. A double strength (3%) agar was prepared. The two parts were autoclaved and the medium of the desired pH was prepared by aseptically mixing the sterile double strength broth into the sterile double strength agar. This gave a medium of ± 0.1 pH of the desired pH level. The medium formulated in the labortory will be designated "Lab Medium" and that derived from the commercial dehydrated material will be designated as Plate Count Agar. The Lab Medium (pH 4.0) was used for the isolation and also for the maintenance of acid tolerant organisms.

Quantitative estimations of microorganisms were made by standard serial dilution plate count procedures. Five plates per dilution were made and no less than three plates per dilution were taken as a quantitative estimation.

---

*Beckman Instruments, Inc., Fullerton, Calif.
**Hach Chemical Co., Ames, Iowa
***Difco Laboratories, Detroit, Michigan

Spread plates (surface inoculation) were also used to quantitatively and qualitatively estimate the number and types of microorganisms present. The medium was first poured into plates and allowed to solidify with the lids slightly ajar to speed drying of the agar surfaces. One milliliter of each dilution (in some instances 0.1 ml) was placed on the agar surface and spread with an L-shaped sterile glass rod. Controls of both methods were used and consisted of one milliliter of a sterile dilution blank plated in the above manner.

The method used for qualitative estimates of microorganisms was to pick ten different colony types from each plate. These were placed into drops of water on glass slides, air dried and either simple or gram stained. In some instances, all colonies present on a plate were expressed as the ratio of bacteria to yeasts or to filamentous fungi, if they were present in significant numbers.

The methods recommended in the Manual of Microbiological Methods (1957) by the Society of American Bacteriologists were closely followed for the identification of the bacterial isolates obtained during the course of this work. Classification was according to Bergey's Manual of Determinative Bacteriology (Breed, Murry and Smith, 1957) Isolates which grew on both acid and neutral media were treated as separate microorganisms for the basic tests, i.e., gram stain, morphology and carbohydrate fermentation. If these were identical they were considered to be the same organism and further identification was carried out using the isolates from neutral medium. Standard inoculation for biochemical and physiological tests, using a liquid medium, was to use a loopful of an 18- to 24-hour plate count broth culture. The same loop was used for all experiments and so remained the standard inoculum. Flagella were stained according to the method of Leifson (1952) and spores by the cold method of Bartholomew and Mittwer (1950).

RESULTS AND DISCUSSION

Three other media were compared with low pH plate count medium for their value is the isolation of microorganisms. These were Czapek Dox Medium (Oxoid) *; Potato Dextrose Agar (Difco) and WL Nutrient Medium (Fisher) **. All were prepared according to the manufacturer's directions, autoclaved, tempered to 45 C and then adjusted to pH 4.0 with 6 N H_2SO_4. Agar (2%) was added to the Czapek Dox Liquid Medi-

*"Oxoid" Division of Oxo Limited, London
**Fisher Scientific, Pittsburgh, Pennsylvania

um as a solidifying agent to adapt it for plate counts. The results are shown in Table 1. Although the results obtained using WL Nutrient Medium were essentially the same as those obtained by the Plate Count Medium, the latter was preferred because the WL Medium was somewhat dark in color and therefore small subsurface and chromogenic colonies were difficult to distinguish.

Survival of Microorganisms

It has been reported that at least some bacteria die whenever a sudden change in environmental pH occurs as when sewage enters an acid mine water polluted stream or acid mine water enters a sewage polluted stream. The magnitude of this effect was investigated.

Methods

(1) A sewage-acid mine water mixture (1:1), pH 3.0-3.1, was made and incubated at 22 C. At hourly intervals during a 12-hour period, the numbers of microorganisms were determined. Plate Count Agar was used for plating. The incubation period was 72 hours at 26± 1 C.

(2) Sixty ml of acid mine water was added to one liter of sewage. The mixture, at a pH of 3.5, was divided into two equal portions, one portion incubated at 4 C and the other at 22 C. At intervals during a 72-hour incubation period the number of viable microorganisms was determined by plating. The plating medium was Plate Count Agar and these plates were incubated at 26± 1 C for 72 hours before counting.

Each of the above experiments was repeated four times. The figures in each case reflect the results from only one experiment.

TABLE 1

Comparison of Four Different Media In Recovery of Microorganisms From Raw Sewage - Acid Mine Water Mixtures At pH 2.9

Media (pH 4.0)	Microorganisms per ml
W L Nutrient Medium	1,300
Czapek Dox Medium + Agar	800
Potato Dextrose Agar	1,000
Plate Count Medium	1,500

(3) In a third experiment, 340 ml of acid mine water were required to adjust one liter of sewage of pH 3.5, which was then divided into two

equal portions, one of which was incubated at 4 C and the other at 22 C. Except for the six-hour count, microbial determinations on each were made at 24-hour intervals. After 144 hours the 4 C portion was changed to 22 C and the 22 C portion to 4 C. Microbial determinations were continued at intervals, using Lab Medium and Plate Count Agar at pH 4 and pH 7, respectively. Incubation was at 25 C.

(4) In a final study of the pH-survival phenomenon of microorganisms, a 1:2 sewage-acid mine water mixture was made. The 2.65 pH mixture was then divided into three equal lots. To one lot a $Ca(OH)_2$ suspension was added to adjust the pH to 3.5. A second lot was adjusted to pH 4.5 with the $Ca(OH)_2$ suspension. The third lot received no $Ca(OH)_2$ and was the control. All were incubated at 27 C for 10 hours. At intervals microbial determinations were made using Plate Count Agar. Incubation was at 27 C for 72 hours.

Results

The data from the first study are presented graphically in Figure 1. The greatest decrease in microbial numbers occurred during the first

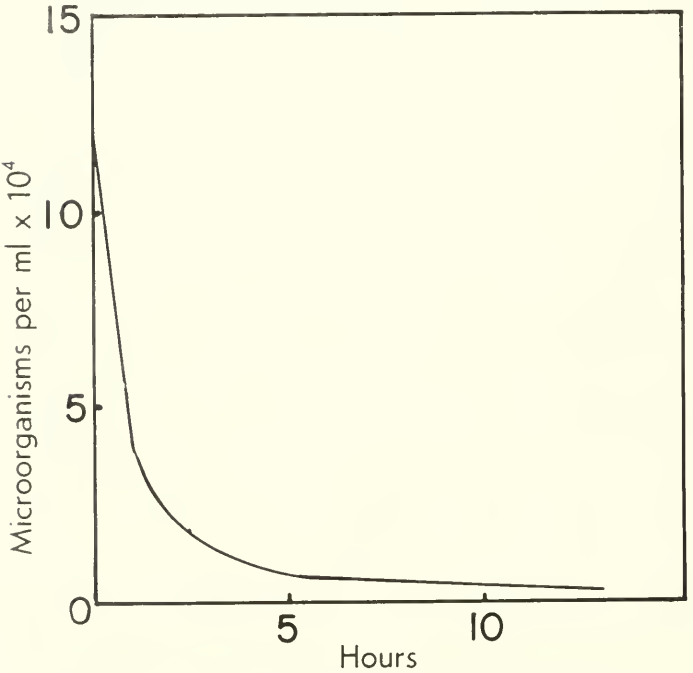

Figure 1. Effects of acid mine water on the number of microorganisms in raw sewage mixed 1:1 with acid mine water. Incubation at 22 C.

hour after the sewage was made strongly acid, after which the rate of decline gradually slowed.

The data from a second study are presented in Figure 2. The results for the first 12 hours at both temperatures (4 C and 22 C) showed the

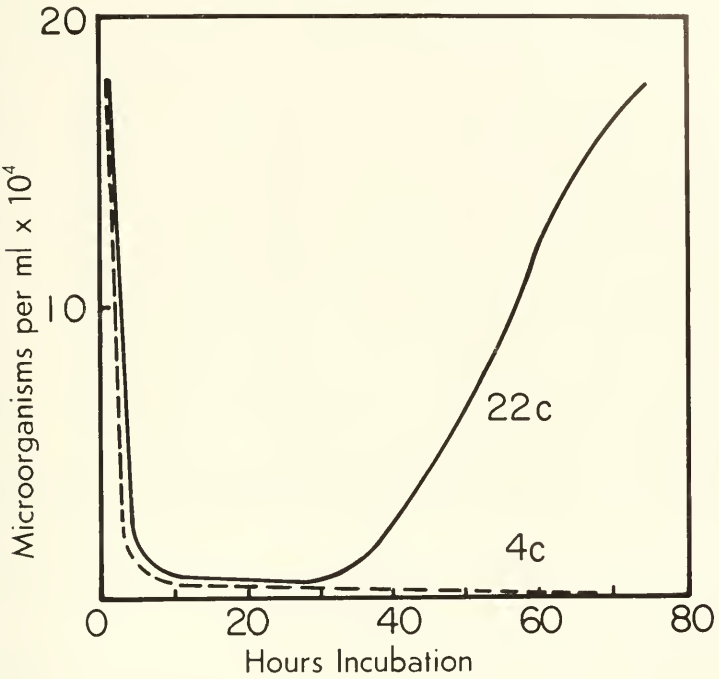

Figure 2. Growth of raw sewage microorganisms at 4 C and 22 C in raw sewage acid mine water mixtures adjusted to pH 3.5

same general reduction in viable cells as those of the first study. The slow but gradual decrease observed in this study between the hours 5 and 12 (Figure 1) continued for approximately 35 hours (incubation at 22 C). The number of microorganisms then began to increase rapidly and was still increasing, although not as rapidly, at the end of the incubation period (72 hours). The material held at 4 C showed a different trend, the numbers of viable microorganisms continuing a slow but steady decrease until the termination of the experiment at 72 hours.

The results obtained from the third study of microbial survival in sewage-acid mine water mixtures at pH 3.5 are presented in Table 2. The microbial numbers in the sample stored at 4 C continued to decrease throughout the entire first part of the test period of 144 hours. After the sample was changed to 22 C at 144 hours, the numbers began

TABLE 2[1]

MICROBIAL SURVIVAL IN SEWAGE-ACID MINE WATER MIXTURES (pH 3.5). INCUBATED AT 4 C AND 22 C

Time (hrs)	Plating Media			
	pH 7 Medium		pH 4 Medium	
	4 C	22 C	4 C	22 C
0	604,000	604,000	1,900	1,900
6	57,600	41,000	834	614
24	41,400	17,100	3,050	3,300
48	31,000	368,000	820	12,860
72	15,080	612,000	478	95,400
96	11,700	712,000	702	566,000
120	7,900	306,000	437	518,000
144	5,460	512,000	432	492,000
		Temperatures Switched		
	22 C	4 C	22 C	4 C
168	3,425	206,800	2,880	380,000
192	5,475	204,800	8,080	360,000
216	29,400	219,400	33,200	200,000
240	181,000	276,200	169,200	340,000
360	674,000	398,000	678,000	878,000

Repetition of run 1, but the flasks were switched after 144 hours. The flask at 22 C was transferred to 4 C and further counts were made for an additional 216 hours. The same treatment was also given the flask originally at 4 C. It required 340 ml of acid mine water to adjust the pH of one liter of sewage to 3.5

to increase and continued to do so until the test was terminated after 216 hours, the total text period was 360 hours.

The microbial numbers in the sample first stored at 22 C reflected the typical decrease previously observed before the numbers increased and reached an uneven stationary phase over the 72-144-hour period. This sample when changed to 4 C at 144 hours showed, in general, only a small decline in microbial numbers.

In the fourth study in which calcium hydroxide was used, the most rapid decrease in microbial numbers occurred in the pH 2.65 mixture (no $Ca(OH)_2$). The rate of decrease was less rapid at pH 3.5 and slowest at 4.5. After 7.5 hours the numbers of survivors were about the same at all three pH levels, and the death rates, until the end of the experiment, were nearly equal. There was some change in the pH levels during the experimental period. The pH of the mixture with no $Ca(OH)_2$ changed from 2.65 to 2.70. The pH 3.5 mixture had a final pH of 3.0 and 4.2 was the final pH of the other mixture. The results are presented in Figure 3.

[1]The authors wish to thank Harold A. Cook, graduate student in the Department of Plant Pathology and Bacteriology for repeating one of the experiments and supplying the data for the table.

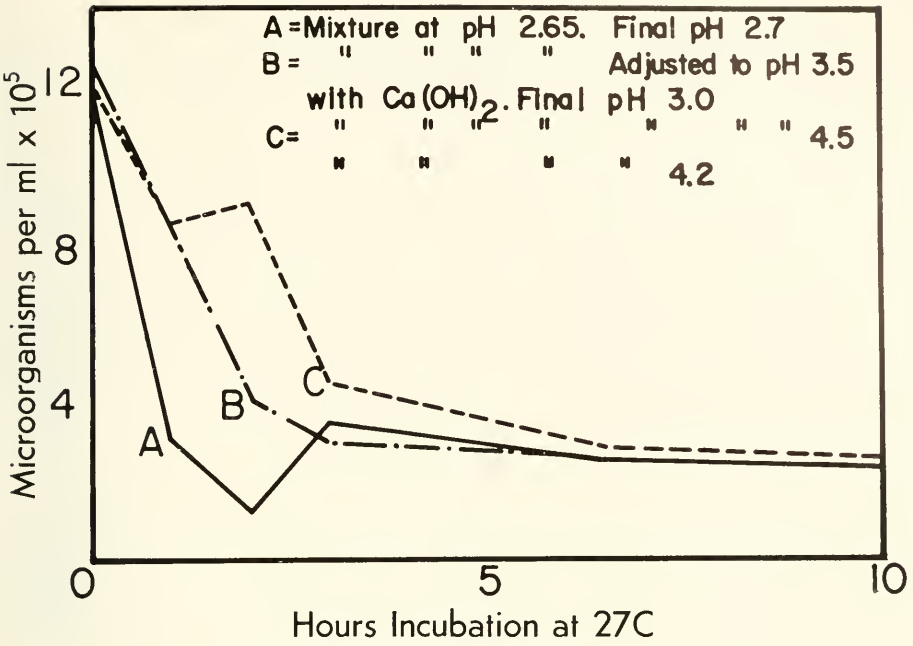

Figure 3. Raw sewage acid mine water mixtures 1:2. Initial pH 2.65

Discussion

Regardless of the quantity of acid mine water added to sewage, if the pH level became strongly acid, a rapid decrease in microbial numbers occurred during the first few hours. Most of it probably occurring within the first hour. Following the initial reduction, the decrease became slow.

Temperature also influenced the results. At low temperature and low pH the number of microorganisms continued to decrease. At 22 C and low pH, following the decrease as indicated above, an increase in numbers occurred. This increase would indicate either a "recovery" of the microorganisms from the pH shock or the increase of a specific group of microorganisms, such as yeasts.

A strongly acid sewage-acid mine water mixture was held at a low temperature for a period of time and then changed to a higher temperature (22 C). An increase in numbers of microorganisms occurred just as was observed in material originally held at 22 C. On the other hand, in the material held at 22 C until the increase in microbial numbers reached a peak and then transferred to a low temperature (4 C), only

a slight decrease in the number of microorganisms occurred. This would indicate that the organisms present, which grew after the initial decrease at low pH, were now only slightly affected by the temperature in the range 4 C to 22 C.

Although the pH of the sewage-acid mine water was adjusted with $Ca(OH)_2$ to pH 3.5 and 4.5 from an initial pH of 2.65, a rapid decrease in microbial numbers occurred. Even at pH 4.5 the decrease continued, and after 10 hours, the number of microorganisms at pH 4.5 was no greater than at pH 2.65 or pH 3.5.

No attempt was made in any of these studies to determine whether the surviving microorganisms were predominately yeasts or bacteria. Previous studies have shown that at low pH levels, yeasts are the predominating organisms, although bacteria are also found.

Selectivity

Previous investigations (Wilson, Hipke, & Rogers, 1965; Rogers and Wilson, 1966) had shown that pH, at least to some extent, is a selective mechanism as to the types of microorganisms predominating in sewage-acid mine water mixtures. This was further investigated by adjusting the pH of the medium to various levels and plating raw sewage.

Method

Lab Medium was adjusted to various pH levels using approximately 6N sulfuric acid. Acid mine water was not used in the pH adjustment because it caused a heavy precipitate to form in the medium. Serial dilutions were made and plated. Incubation was at 22 C for 72 hours. After the colonies developed on the medium they were examined and classed as bacteria, yeasts or filamentous fungi.

Results

The total number of microorganisms, as previously shown, decreased as the pH decreased. The data also showed a change in microbial types as pH decreased. As the pH of the environment changed from pH 7.0 to less than pH 3.0, the predominating organisms changed from bacteria to yeasts to filamentous fungi. These changes are presented diagrammatically in Figure 4.

Discussion

The major change in the predominating flora seems to occur in three areas. One is above the pH 4.5-5.0 range, another between this range and

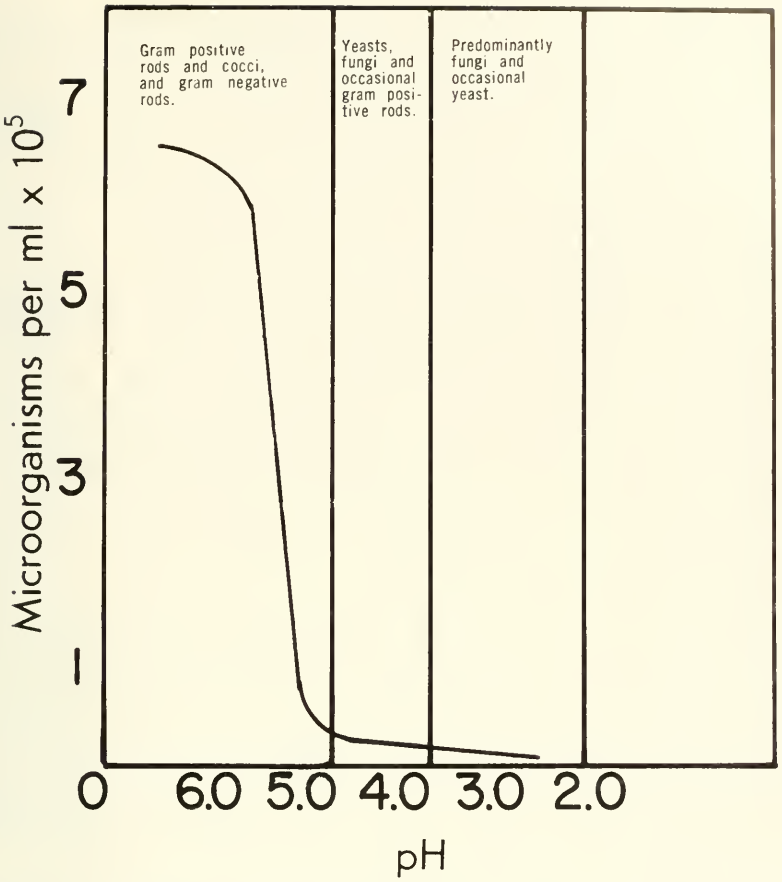

Figure 4. The numbers and types of microrganisms recovered from raw sewage using a medium adjusted to various pH levels.

about pH 3.5, and the third region below pH 3.5. The divisions between these areas are not absolute. The changes in microorganisms varied from one sample to another, but the transition zones occurred around these pH levels. Consequently, these three areas were rather distinct and sewage breakdown in each area was carried out primarily by a different group or groups of microorganisms.

Oxygen Demand

Sewage decomposition is an activity of microorganisms, and one of the easiest methods of measuring the microbial activity in sewage decomposition is to measure the utilization of oxygen by the microorganisms within the system.

A series of studies was made to determine the oxygen demand of microorganisms in: (1) sewage-acid mine water mixtures at various pH levels; (2) sewage-sulfuric acid mixtures at various pH levels; (3) sewage-acid mine water mixtures in which NaOH was used to adjust the pH levels; and (4) sterile sewage-sterile acid mine water mixtures inoculated with two bacteria and/or two yeasts.

Each series of experiments will be discussed separately.

Influence of pH Upon Oxygen Demand

Since pH exerts an influence upon microbial activity this influence can be measured by determining the oxygen demand.

Method. — The 5-day oxygen demand of sewage-acid mine water mixtures was determined using the Hach apparatus manometric method with readings recorded daily. The amount of sewage was constant at all pH levels. In those samples (controls) which received no acid mine water, an equal amount of distilled water was added. The initial pH of the mixtures was 7.5, 5.0, 4.5, 4.0, and 3.5. The pH at the end of the 5-day period also was determined.

Results. — The sewage-distilled water mixture (control), pH 7.5, had the greatest oxygen demand and the sewage-acid mine water mixture, pH 3.5, had the least demand. The oxygen demand at pH 4.0, 4.5, and 5.0 was about equal; each greater than that of pH 3.5 but considerably less than that at pH 7.5. At all pH levels except 7.5, the demand for oxygen was slow for the first 25-60 hours and then increased. At pH 3.5, the increase in the rate of oxygen utilization began between two and three days after the start; at pH 4.0, 4.5, and 5.0, it began after one day of incubation. Results are presented in Figure 5.

Effects of Acid Mine Water and Sulfuric Acid on Raw-Sewage Microorganisms

There have been indications that some factor other than pH has had an influence on the microorganisms in sewage-acid mine water mixtures. An attempt was made to gain some insight into this possibility by comparing the oxygen utilization in sewage-acid mine water mixtures with sewage diluted with distilled water and the pH adjusted wth sulfuric acid.

Method. — Sewage was diluted with phosphate buffer dilution water, made according to the directions in Standard Methods (1960). The mixture was divided into several equal portions and the pH adjusted either with acid mine water or sulfuric acid. Standard 300 ml BOD bottles were then filled with the adjusted mixtures.

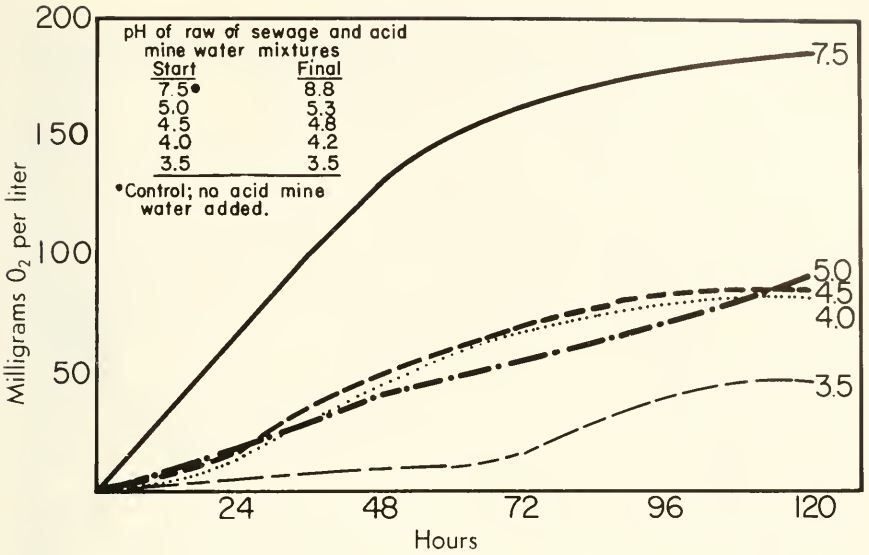

Figure 5. Oxygen demand of raw sewage - acid mine water mixtures at various pH levels.

Whenever a determination was to be made, the bottle was placed on a magnetic stirrer, its stopper removed, and the oxygen sensor inserted, making a tight seal between sensor and bottle neck. Care was taken that no air bubble was trapped on the end of the sensor. After a determination was made, the sensor was removed, the stopper carefully replaced, and the bottle returned to the 20 C BOD incubator.

The results are not expressed as BOD values, inasmuch as the information desired was the oxygen demand trend of the mixtures.

Results. — The data are presented in Figures 6 and 7. The amount of oxygen utilized decreased as pH decreased, regardless of whether acid mine water or sulfuric acid was used to adjust the pH level, particularly at pH 4.5 and pH 3.5. The utilization was more rapid with the sulfuric acid adjusted samples at these two levels.

Effect of Partial Neutralization of Acid Mine Water Upon Oxygen Demand

Many sewage microorganisms are killed quickly if sufficient acid mine water is added to sewage to lower the pH below pH 4.0. To determine the effect upon the oxygen demand, a study was made using acid mine water which had been completely or partially neutralized.

Methods. — Sewage-acid mine water was mixed in a 1:1 ratio. The resulting pH was 3.1. The mixture was divided into equal parts and the

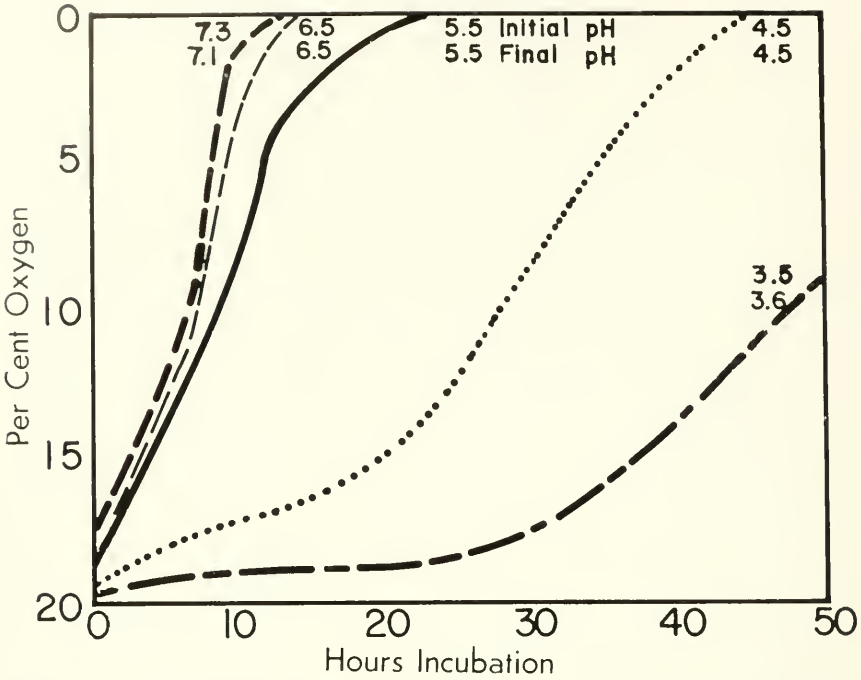

Figure 6. Oxygen utilization by sewage microrganisms in raw sewage-acid mine water mixtures at various pH levels.

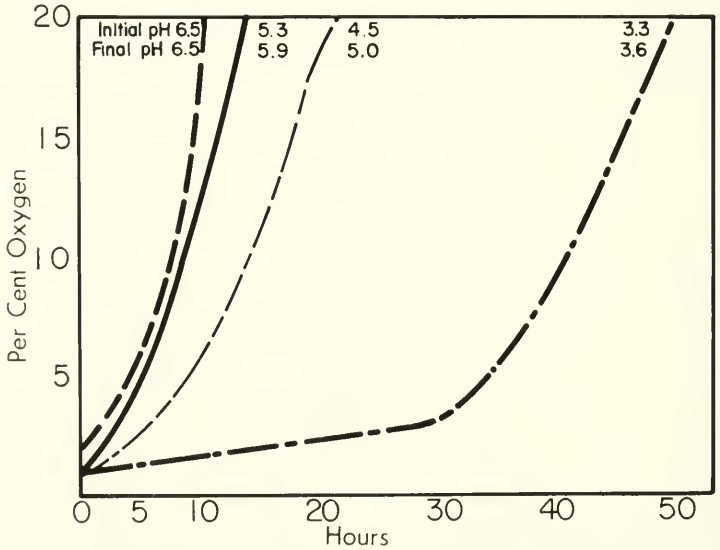

Figure 7. Oxygen utilization by sewage microrganisms in raw sewage - H_2SO_4 mixtures at various pH levels.

pH adjusted to various levels with 1N NaOH. The pH levels were: 7.3, 6.0, 5.0, and 3.2. A 1:1 sewage-distilled water mixture which had a pH of 7.3 served as the control. The manometric apparatus was used to follow the oxygen demand of the mixtures. Incubation was in a 20 C BOD incubator.

Results. — The greatest oxygen demand was by the control, pH 7.3, which received no acid mine water. Its oxygen demand was considerably greater than the 1:1 sewage-acid mine water mixture adjusted to pH 7.3. In these studies, as in others, the oxygen demand decreased as the pH decreased. The results are presented in Figure 8.

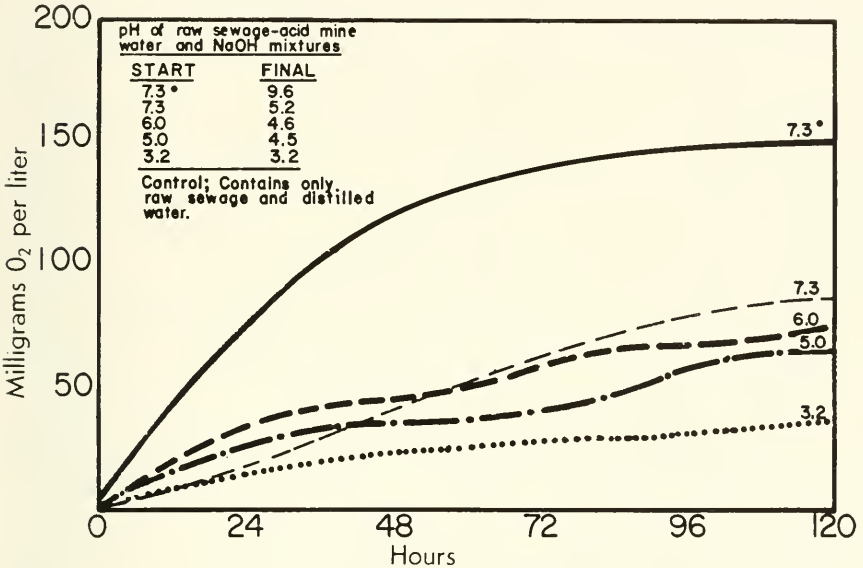

Figure 8. Oxygen uptake of raw sewage - acid mine water and NaOH mixtures at various pH levels.

Methods. — In the above experiment the bacteria in the sewage were subjected to the "pH shock," which apparently kills many bacteria. A second experiment was designed to eliminate this shock by neutralizing the acid mine water before adding it to the sewage. The mixtures used, and the initial and final pH values are presented in Figure 9.

Results. — The results are presented in Figure 9. The pH 7.2 control that contained no acid mine water had a 5-day oxygen demand slightly less than another sample (pH 6.8) which contained some acid mine water. Both of these samples had a final pH (after 5 days) of 9.7. The least oxygen demand was from a 1:1 sewage acid mine water mixture which

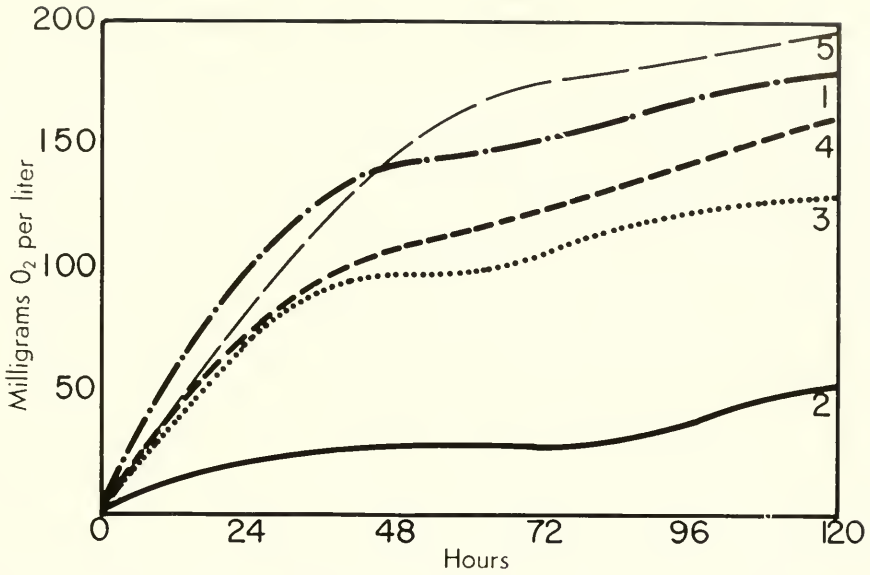

Figure 9. Comparison of the effects of neutralized and unneutralized acid mine water on the BOD of raw sewage.

Bottle No.	pH			Raw Sewage Milliliters	Acid mine water (pH 2.7) Milliliters	Acid mine water (pH 7.4)[b] Milliliters	Distilled Water Milliliters
	Start	Final	Projected[a]				
1	7.2	9.7	- - -	100	00	50	50
2	3.4	4.1	3.4	100	00	00	100
3	7.0	7.8	3.4	100	100	00	00
4	6.8	7.8	3.8	100	00	100	00
5	6.8	9.7	4.4	100	00	25	75

[a]The pH that would result from the use of acid mine water of pH 2.7.
[b]Acid mine water neutralized with 1N NaOH.

had beginning and final pH values of 3.4 and 4.1 respectively. A 1:1 sewage-neutralized acid mine water mixture had a considerably greater 5-day oxygen demand than the sewage-acid mine water mixture. The beginning and final pH values in this case were 7.0 and 7.8 respectively. The sample would have had a pH of 3.4, as the previous sample, had the mine water not been neutralized. A 2:1:1 sewage-neutralized acid mine water-distilled water mixture had an oxygen demand slightly less than the control (sewage-distilled water).

Oxygen Demand Using Bacteria or Yeasts

The previous studies of oxygen uptake involved the mixed population in the raw sewage. In this study sterile sewage and sterile acid mine water were inoculated with bacteria and/or yeasts.

Methods. — Sewage (130 ml) was dispensed into each of five Hach BOD Apparatus bottles, then capped and autoclaved. After sterilization, 50 ml of sterile distilled water was added to each of two bottles. The pH was 9.0. One of these bottles was inoculated with bacteria and yeasts, the other with bacteria only. The three remaining bottles each received 50 ml of filter sterilized acid mine water. The resulting pH was 3.5. One bottle was inoculated with bacteria and yeasts, a second with bacteria and a third with yeasts.

The two bacteria (A-27, A-31) and the two yeasts (A-2, A-12), used as inocula had been isolated from an acid environment. One ml of a 24-hour broth culture of each organism was used as inoculum. More details of the experimental conditions are shown in Figure 10.

Results. — The data are presented in Figure 10. Sterile sewage at pH 9.0 had the greatest oxygen demand when inoculated with both yeasts and bacteria. The oxygen demand was considerably lower when only the two bacteria (A-27, A-31) constituted the inoculum. Acid sewage (pH 3.5) had a higher oxygen demand when inoculated with bacteria and yeasts than with bacteria alone. Acid sewage (pH 3.5) inoculated with the yeasts had a higher oxygen demand than similar sewage inoculated with the bacteria only, but it was less than that of the pH 3.5 sewage containing both bacteria and yeasts.

Discussion. — The oxygen demand of sewage mixed with acid mine water or sulfuric acid decreased as the pH decreased. This was in agreement with the utilization of glucose in sewage-acid mine water mixtures as reported by Wilson, Hipke and Rogers (1965). In all of the results a general trend was evident: a longer lag period was required at the lower pH levels before the rate of oxygen utilization was of any substantial

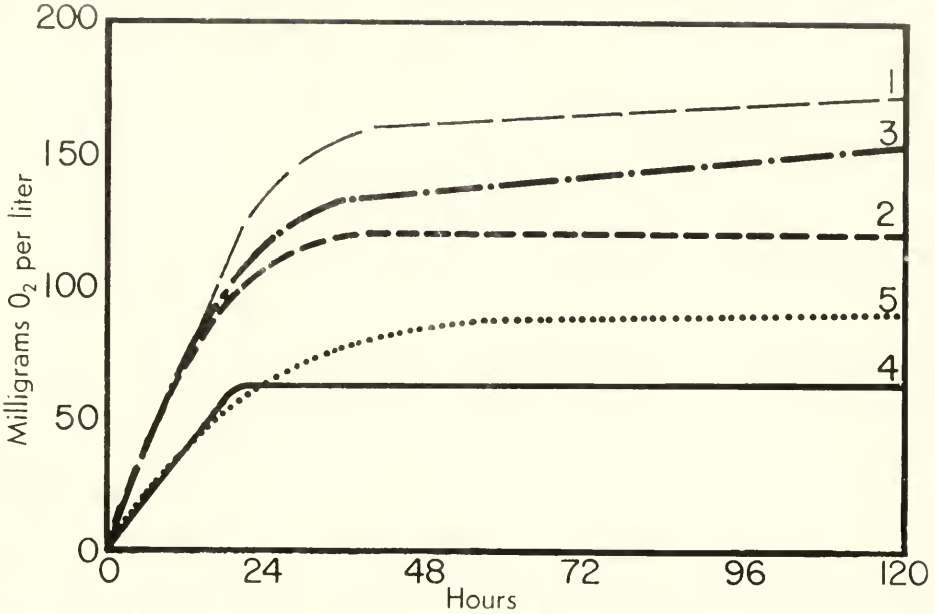

Figure 10. Comparison of BOD values obtained in sterile raw sewage-acid mine water mixtures inoculated with acid tolerant yeasts and bacteria.

Mixtures No.	pH		Milliliters of distilled	Milliliters of acid mine	Milliliters of plate count broth cultures			
					Bacteria		Yeasts	
	Start	Final	water	water	A-27	A-31	A-2	A-12
1	9.0	10.7	50	00	1.0	1.0	1.0	1.0
2	9.0	11.2	50	00	1.0	1.0	0.0	0.0
3	3.5	4.9	00	50	1.0	1.0	1.0	1.0
4	3.5	4.7	00	50	1.0	1.0	0.0	0.0
5	3.5	4.5	00	50	0.0	0.0	1.0	1.0

magnitude. This was probably due to a change in the predominating microorganisms. Although yeasts are found in sewage, they do not appear to dominate the sewage microbial population until the pH is 4.5 or below. A period of time, therefore, is required before the number of yeasts is great enough to be reflected in the utilization of oxygen.

Although the oxygen demand decreased as the sewage pH decreased, whether by acid mine water or sulfuric acid, a difference in the oxygen utilization at the same pH level did exist. The idea that some factor other than pH in acid mine water influences the microorganisms in a sewage-acid mine water mixture has been previously presented. These data supported the idea, but unfortunately were not complete enough to offer unqualified proof or even to suggest what another factor might be.

The data on survival of microorganisms in sewage-acid mine water mixtures suggested that many microorganisms were killed within a short time. If the mixtures were neutralized immediately, little or no difference was apparent. The results using NaOH as a neutralizing agent following the sewage-acid mine water mixture were inconclusive because of the pH change during the five-day incubation period. More conclusive results were obtained by the use of neutralized acid mine water added to the sewage.

Little difference occurred in the oxygen demand of a sewage-distilled water mixture and a sewage-neutralized acid mine water mixture of the same final (five-day incubation) pH. On the other hand, a 1:1 sewage-neutralized acid mine water mixture (pH 7.8) had considerably less oxygen demand than mixtures with less acid mine water, although the quantity of sewage was equal.

The data obtained indicated that a combination of bacteria and yeasts inoculated into sterile sewage-acid mine water mixtures results in a greater oxygen demand at both pH 9.0 and pH 3.5 (pH 10.7 and pH 4.9 after five days incubation) than bacteria alone at pH 9 and yeasts alone at pH 3.5. Bacteria alone had a rapid oxygen utilization rate at pH 3.5 (final pH 4.7) for the first 24 hours, as did the other inoculated mixtures, but no additional oxygen demand during the remaining four days of incubation. This would indicate changes in availability for the organisms of certain constituents of the sewage resulting from the autoclaving. It is conceded that equal amounts of inoculum should have been used. For example, using only bacteria or yeasts two milliliters was the quantity of inoculum (1 ml each) whereas, when the inoculum was both, the total quantity of inoculum was four milliliters. This however, is probably unimportant in these data, inasmuch as the maximum oxygen

demand was reached in less than 48 hours in all combinations, with one exception (Fig. 10).

Microbial Isolates and Observations

Isolates

In addition to the survival studies, some quantitative and qualitative studies were made upon the microorganisms in sewage-acid mine water mixtures.

Methods. — Monongahela River water was collected at two locations and standard plate count determinations of the microorganisms were made, using both the pour plate technique and spread (surface inoculation) plate technique. Plate Count Agar (pH 6.7) and Lab Medium (pH 4.0) were used. The plates were incubated at 25 C for 72 hours.

Results. — The data are presented in Table 3. The pH of the river water varied from 4.2 to 5.3. As many bacteria were found at pH 4.2 as at pH 5.3. The data do show the selectivity of Lab Medium (pH 4.0) for yeasts. Large numbers of bacteria, but few yeasts were found on Plate Count Agar (pH 6.7). A few filamentous fungi grew on the Lab Medium (pH 4.0).

TABLE 3

QUANTITATIVE AND QUALITATIVE ESTIMATES OF MICROORGANISMS FOUND IN MONONGAHELA RIVER WATER

Month/year Sample Collected	pH	Plate Count Agar pH 6.7, microorganisms	Ratio of bacteria to yeasts to filamentous fungi	Plate Count medium pH 4.0, microorganisms	Ratio of bacteria to yeasts to filamentous fungi
		x 10^3/ml		x 10^3/ml	
12/65	4.2	30.0[a]	7:0:0	0.05[a]	0:6:1
12/65	4.2	0.9[a]	9:0:0	0.13[a]	0:7:1
12/65	4.3	7.3[a]	9:0:0	0.09[a]	0:2:1
4/66	5.4	16.0[b]	10:0:0	0.07[b]	3:2:0
4/66	5.4	73.0[b]	10:0:0	0.14[b]	2:3:0

[a]Pour plate technique
[b]Smear plate technique
[c]All colonies on plates picked

Methods. — Isolations were made from sewage-acid mine water mixtures. All isolates were isolated either from an acid mine water environment or, if from another source, they grew on a medium of pH 4.0. The isolates were classed as bacteria, yeasts, or filamentous fungi.

Results. — Fifteen of the isolates were bacteria, 26 were yeasts and 16 were filamentous fungi. Of the bacteria, only one was a gram negative rod, two were gram positive cocci, and the rest were gram positive rods. Some died in storage before their identification was begun and others lost their acid tolerance. A few of the latter regained their acid tolerance upon repeated transfer, but the investigations were concluded before these could be identified. The identity of the remaining seven was established.

Four of the seven isolates belonged to the genus *Bacillus.* Positive species identification was not successful. They were either *B. coagulans* or *B. firmus,* but characteristics were enough different from the descriptions in Bergey's Manual (Breed and others, 1957) to place either choice in doubt. Of the three remaining isolates, one closely resembled *Micrococcus candidus* but differed in the fermentation of carbohydrates. Of the two remaining rods, both gram positive, one was identified as *Kurthia variabilis;* the other could not be identified even to genus.

Observations

Some investigations of the microorganisms were also made either by microscopical examination of stained slides of sewage mixtures used in other studies or by serial dilution standard plate count procedures.

Microscopic examination of stained smear slides revealed essentially what was previously noted by Wilson, Hipke and Rogers (1965). Bacteria predominated on slides made of approximately neutral sewage mixtures. As the pH of the mixtures decreased the numbers of yeasts increased. At pH 3.5 neither yeasts nor bacteria seemed to predominate when stained smears were microscopically examined, but pour plates from the same mixtures did reveal that yeasts predominated at the lower pH levels. The results are shown in Table 4.

The numbers of microorganisms were determined by the standard plate count procedures from the sewage-acid mine water mixtures used to determine the oxygen demand (Figure 5). The plates were incubated at 20 C for five days. In addition to the counts, random colonies were picked from the plates, microscopic smears made and examined to determine whether the colonies were bacteria or yeasts. The numbers of microorganisms in sewage mixtures between pH 7.5 and pH 3.5 decreased as the pH decreased. The numbers in mixtures at pH 5.0, 4.5, and 4.0 were nearly equal (Table 5), as was reflected by the oxygen demand curves of the same mixtures (Figure 5).

TABLE 4

QUANTITATIVE AND QUALITATIVE ESTIMATE OF MICROORGANISMS IN RAW SEWAGE-ACID MINE WATER AND RAW SEWAGE-H_2SO_4 MIXTURES AT pH 3.5 AND CONTAINING LESS THAN 0.5 PER CENT OXYGEN AFTER 50 HOURS INCUBATION AT 20 C.

Medium[a] pH	x 10^4/ml	Ratio of bacteria to yeasts
Raw Sewage-acid mine mixture		
6.7	13.0	0:10
4.0	17.0	0:10
Raw Sewage-H_2SO_4 mixture		
6.7	65.0	2:8
4.0	57.0	0:10

[a]Plate counts were made on media at two different pH levels.

Discussion. — The pH of the medium used in the estimation or isolation of microorganisms from sewage-acid mine water mixtures can influence results. Media at a pH near neutral favored the growth of bacteria over yeasts, but the reverse was true if the pH was 4.0. This would indicate that some bacteria are not killed immediately at low pH levels resulting from acid mine water but may be in a bacteriostatic condition. When these bacteria are plated on neutral medium they again multiply.

TABLE 5

NUMBERS OF MICROORGANISMS IN RAW SEWAGE-ACID MINE WATER MIXTURES AT VARIOUS pH LEVELS AFTER 120-HOURS INCUBATION AT 20 C

pH of mixture	Plate Count Agar pH 7.0 microorganisms x $10/^4$ml	Ratio of bacteria to yeast	Plate Count medium pH 4.0 microorganisms x 10^4/ml	Ratio of bacteria to yeast
7.5[a]	884	10:0	0.08	0:10
5.0	300	10:0	23	1:9
4.5	300	5:0	30	3:1
4.0	320	4:0	[b]	4:1
3.5	115	[c]	32	0:10

[a]Control containing only raw sewage and distilled water
[b]Most of the plates were contaminated by fungi
[c]No slides were made

The identification of the acid-tolerant bacteria has not been productive. Rogers and Wilson (1966) reported an *Aerobacter aerogenes* iso-

late and an *Escherichia* sp. that grew at pH 4.0. In the present studies, four isolates that grew at pH 4.0 were identified as *Bacillus* species, probably either *B. coagulans* or *B. firmus,* or closely related to them. One isolate was tentatively identified as *Micrococcus candidus,* one as *Kurthia variabilis.* Evidence indicates that the active bacteria in sewage-acid mine water mixtures which will grow at pH 4.0 probably represented only a few bacterial genera.

In this work, no attempt was made to identify the yeasts.

CONCLUSIONS

1. If sufficient acid mine water enters a sewage-polluted stream to lower the pH to 3.5 or below, a marked decrease in sewage microorganisms will occur within the first hour or so. If the atmosphere and water are warm, the numbers of microorganisms will, within a few hours, begin to increase. Yeasts will probably predominate, however, if the pH remains strongly acid. On the other hand, if air and water temperatures are low, microbial numbers will probably remain small until the water warms.

2. The data indicated that many of the sewage microorganisms could not tolerate the shock of a sudden change in pH from neutral to strongly acid. If this is true, then sewage decomposition in streams polluted with acid mine water is much slower than under neutral conditions, is not complete, and is carried out by organisms other than those found under neutral conditions. This situation would also likely occur even if the sewage-acid mine water polluted stream were neutralized with calcium hydroxide (or carbonate) until it received some additional sewage or other material which would serve as an inoculum.

3. Oxygen utilization by sewage-decomposing microorganisms in acid mine water is influenced by the pH. As the pH decreases, the initial lag in oxygen utilization increases. Whenever the acid-tolerant population increases sufficiently in numbers, utilization increases. Within a short period of time, five days, for example, the amount of oxygen utilized generally will not equal that utilized by sewage at neutral pH. Although it is unlikely to occur naturally, sewage decomposition in an acid mine water stream probably would be slower if only bacteria are present rather than a mixed population of bacteria and yeasts. With a yeast population, oxygen utilization will be greater than with a bacterial population but not so great as when both groups of microorganisms are present.

4. If acid mine water is neutralized before sewage is added, the effect of the mine water upon the sewage microorganisms in minor, which may account for the interpretation of some data that a factor other

than pH in acid mine water exerts a detrimental influence upon the microorganisms. If, however, neutralization occurs after the sewage is added, some decrease in microbial population will occur, a decrease somewhat correlated with the time lapse between the moment of mixing of the sewage and acid mine water and the time of neutralization. The results indicate that the greatest decrease in the microbial population will occur during the first hour or so. Population decrease is reflected in the oxygen utilization of the sewage microorganisms which, in turn, is a measure of the decomposition of the sewage.

5. Under strongly acid conditions, the bacteria involved in sewage decomposition probably are limited to a few species that are acid tolerant strains of normally non-acid tolerant species.

6. The pH of the medium used to isolate microorganisms from a sewage-acid mine water mixture will influence the type of isolates obtained. If the medium is pH 4.0, for example, the isolates will be predominately yeasts. If the medium is pH 7.0, then bacteria are likely to be the predominating organisms isolated, especially if the number of aciduric bacteria is large.

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